Research Note

A Molecular View of the Superfamily Dioctophymatoidea (Nematoda)

Anson V. A. Koehler,[1,3] Eric P. Hoberg,[2] Fernando Torres-Pérez,[1] and Joseph A. Cook[1]

[1] Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131, U.S.A.
(e-mails: ansonkoehler@gmail.com, ftorresp@gmail.com, cookjose@unm.edu) and
[2] U.S. National Parasite Collection, Animal Parasitic Diseases Laboratory, U.S. Department of Agriculture,
Agricultural Research Service, Beltsville, Maryland 20705, U.S.A. (e-mail: ehoberg@anri.barc.usda.gov)

ABSTRACT: Monophyly of the superfamily Dioctophymatoidea was assessed based on analyses of DNA sequence variation among 3 of 4 constituent genera (5 species). Represented is the first molecular phylogenetic evaluation of the Dioctophymatoidea using maximum parsimony, maximum likelihood, and Bayesian inference of 18S nuclear DNA (786 base-pair [bp] segment) and mitochondrial cytochrome oxidase 1 (293 bp) genes. Dioctophymatoidea is monophyletic and includes a clade with *Dioctophyme renale* and *Eustrongylides ignotus* (Dioctophymatidae) as the sister of *Soboliphyme baturini*, *Soboliphyme jamesoni*, and *Soboliphyme abei* (Soboliphymatidae). Within Soboliphymatidae, *S. baturini* is the sister of *S. jamesoni* and *S. abei*.

KEY WORDS: nematode, Dioctophymatoidea, *Dioctophyme renale*, *Eustrongylides ignotus*, *Soboliphyme baturini*, *Soboliphyme jamesoni*, *Soboliphyme abei*, phylogenetic, cytochrome oxidase 1, 18S.

The dioctophymatoids (order Dioctophymatida, superfamily Dioctophymatoidea) are an enigmatic group of nematodes characterized morphologically by well-developed multipolar cells connecting the body wall and the intestine, 8 longitudinal striae, and the presence of a caudal bursa in males (Karmanova, 1986). All dioctophymatoids are gastrointestinal parasites of birds and mammals as adults, and they utilize oligochaetes as intermediate hosts (Anderson, 2000). Molecular tests of monophyly of dioctophymatoids have been lacking. Dioctophymatoidea is regarded as a basal group within the Nematoda based primarily on morphological criteria (Karmanova, 1986). Among the dioctophymatoids (Rusin et al., 2003), the phylogenetic relationship of a single species, *Soboliphyme baturini* Petrov, 1930, has been explored using the nuclear small-subunit ribosomal RNA gene (18S) and morphological characters. That study supported a sister-group relationship with the Trichocephaloidea (*Trichinella spiralis* (Owen, 1835) and *Trichuris muris* Schrank, 1788).

As currently constituted, the Dioctophymatoidea is composed of 2 families, Soboliphymatidae, including 9 species in the genus *Soboliphyme* Petrov, 1930, and the Dioctophymatidae including the monotypic genus *Dioctophyme renale* (Goeze, 1782), 11 species in the genus *Eustrongylides* Jagerskiold, 1909, and at least 6 species in the genus *Hystrichis* Dujardin, 1845. Species of *Soboliphyme* are primarily stomach-dwelling nematodes of insectivores throughout Eurasia and North America, and 1 species, *S. baturini*, is found chiefly in mustelids (Ribas and Casanova, 2004). *Dioctophyme renale* (giant kidney worm) is found primarily in the kidneys of mink (*Neovison vison* [Schreber]), and other carnivores throughout the world (Measures, 2001). Species of *Eustrongylides* and *Hystrichis* are inhabitants of the proventriculus in avian hosts and are known to be responsible for large mortality events throughout Eurasia and North America (Cole, 1999).

In this phylogenetic study, we use DNA sequences of 5 dioctophymatoids and 3 Trichocephaloidea and include the outgroup *Xiphinema americanum* as identified in broader analyses (e.g., Blaxter et al., 1998; Rusin et al., 2003; Meldal et al., 2007). We test whether the Soboliphymatidae (*Soboliphyme* spp.) and Dioctophymatidae (*Dioctophyme* and *Eustrongylides*) are reciprocally monophyletic within the Dioctophymatoidea. Molecular data are used for the first time to test current assumptions of monophyly of Dioctophymatoidea (*Dioctophyme*, *Eustrongylides*, and *Soboliphyme*) derived from previous interpretations of morphological data (Karmanova, 1986).

Specimens of nematodes are as follows: (1) Adult *D. renale* (*n* = 1), collected by G. H. Parker, Laurentian University, Ontario, and preserved in 95% ethanol after extraction from the kidney of an American mink (*N. vison*). (2) Larval *Eustrongylides ignotus* (*n* = 1) from western mosquito fish (*Gambusia affinis* [Baird and Girard, 1853]) supplied by E. Marsh-Matthews of the Sam Noble Oklahoma

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[3] Corresponding author. Current address: Department of Zoology, University of Otago, 340 Great King Street, Dunedin 9016, New Zealand.

Table 1. Nematode specimens obtained through the Beringian Coevolution Project (BCP), collaborators, and GenBank used in the assessment of the superfamily Dioctophymatoidea. Included are the host, locality, collection year (if known), MSB accession number, and GenBank accession numbers.

Taxon ID	Host	Location	Collection year	MSB #	18S Accession #	COI Accession #
Soboliphyme baturini 1	*Martes caurina*	Vancouver Island, Canada	2005	NK128176	EU394725	EU128176
S. baturini 2	*M. americana*	Fairbanks, Alaska	unknown	NK128108	EU394726	EF519532
S. baturini 3	*M. zibellina*	Kamchatka, Russia	unknown	NK159573	EU394727	EU394161
Soboliphyme jamesoni 1	*Sorex tundrensis*	Yakutsk, Russia	2006	NK139168	EU394728	EF519533
S. jamesoni 2	*S. roboratus*	Yakutsk, Russia	2006	NK139584	EU394729	EF519534
Soboliphyme abei	*S. unguiculatus*	Hokkaido, Japan	2003	NK159581	EU394730	EF519535
Dioctophyme renale	*Neovison vison*	Sudbury, Ontario, Canada	2005	NK159579	EU394731	EU394733
Eustrongylides ignotus	*Gambusia affinis*	Norman, Oklahoma	2005	NK159580	EU394732	NA
Trichinella britovi	*Rattus norvegicus*	Isola del Dran Sasso, Italy	1985	NA	AY851257	DQ007892
Trichinella murrelli	*Ursus americanus*	Pennsylvania	1982	NA	AY851259	DQ007894
Trichinella nativa	*U. maritimus*	Svalbard, Norway	1984	NA	AY851256	AB252966
Xiphinema index	NA	NA	NA	NA	AM086679	AY382608

Museum of Natural History. (3) Adult *S. baturini* ($n =$ 3) from North American marten (*Martes caurina*, *Martes americana*) and Asian sable (*Martes zibellina*) through the efforts of the Beringian Coevolution Project (BCP) (Hoberg et al., 2003; Cook et al., 2005) and N. Tranbenkova of the Kamchatka Institute of Ecology and Nature Management. (4) Adult *Soboliphyme abei* (Asakawa et al., 1988) ($n = 1$) from the stomach of a long-clawed shrew (*Sorex unguiculatus* Dobson), provided by M. Asakawa of Rakuno Gakuen University, Japan. (5) Adult *Soboliphyme jamesoni* Read, 1952 ($n = 2$) from *Sorex tundrensis* Merriam and *Sorex roboratus* Hollister near Yakutsk, Russia, in the summer of 2006. Each nematode was subsampled from the midsection of the body for molecular sequencing, whereas the head and tail of individuals were archived as physical vouchers deposited in the Museum of Southwestern Biology (MSB) (Table 1). Specimens were frozen or stored in either 70% or 95% ethanol.

Ethanol-preserved specimens were prepared for extraction by soaking in a water bath for 30 min followed by a 10 min spin in a vacuum centrifuge. Total genomic DNA was extracted from individual worms using a commercial kit (AquaPure Genomic DNA isolation kit, Bio-Rad Laboratories, Hercules, California). A 768 base-pair (bp) region of 18S was amplified using primers Sobo18SFWD 5′TTTGG TTTTCGGATCTGAGG-3′ and Sobo18SREV 5′GTAC AAAGGGCAGGGACGTA-3′ (modified from Rusin et al., 2003; GenBank sequence no. AY277895). A 293 bp region of cytochrome oxidase 1 (COI) was amplified with the primers SoboCO1F 5′GCTCAGCTTCGGACA GTTTC 3′ and SoboCO1R 5′TCATGCAAATGAACA TCTAGGG 3′ (Tran, 2003). We were unable to sequence COI for *E. ignotus*.

Total volume for polymerase chain reaction (PCR) was 25μl, consisting of: 14.25 μl of H$_2$O, 1 μl of 10 μM primer each, 1 μl of DNA template ($\sim$5 ng/μl), 2.5 μl each of 25 mM MgCl$_2$, 10 mM deoxynucleotide triphosphates dNTPs, and 10X PCR Buffer II, and 0.25 μl Taq (5 units/μl, Amplitaq$^{®}$). PCR analyses were run on PTC-200 thermocyclers (MJ Research) with the following parameters: initial denaturation at 94°C for 60 sec, subsequent denaturation for 30 sec, annealing at 53°C for 15 sec, and extension of 72°C for 30 sec. These steps were repeated for an additional 34 cycles, followed with a final extension of 72°C for 10 min. Product was visualized via electrophoresis on a 0.8% agarose gel and cleaned using 30% poly-ethylene glycol (PEG) and a QiaQuick$^{®}$ (Qiagen Inc.) cleanup kit. BigDye$^{®}$ Terminator v. 3.1 (Applied Biosystems) was used for cycle sequencing reactions. Excess dyes and primer were removed using Sepha-dex$^{®}$ G-50 spin columns or sodium acetate ethanol wash (Applied Biosystems). Forward and reverse strands were sequenced using an ABI PRISM$^{®}$ 3100 Genetic Analyzer. Sequences were aligned using ClustalW (Chenna et al., 2003). Sequences were deposited in GenBank (Table 1).

Xiphinema americanum Thorne and Allen, 1950 (Longidoridea) (GenBank AM086679 for 18S, AY382608 for COI) was used to root the trees because the genus has been recovered in a sister clade to the Trichocephaloidea and the Dioctophymatoidea (Rusin et al., 2003; Meldal et al., 2006). Since Trichocephaloidea is regarded as the putative sister of the dioctophymatoids (Rusin et al., 2003), species of *Trichinella* (GenBank accession number for 18S, COI), *Trichinella britovi* Pozio, La Rosa, Murrell, and Lichtenfels, 1992 (AY851257, DQ007892), *Trichinella nativa* Britov and Boev, 1972
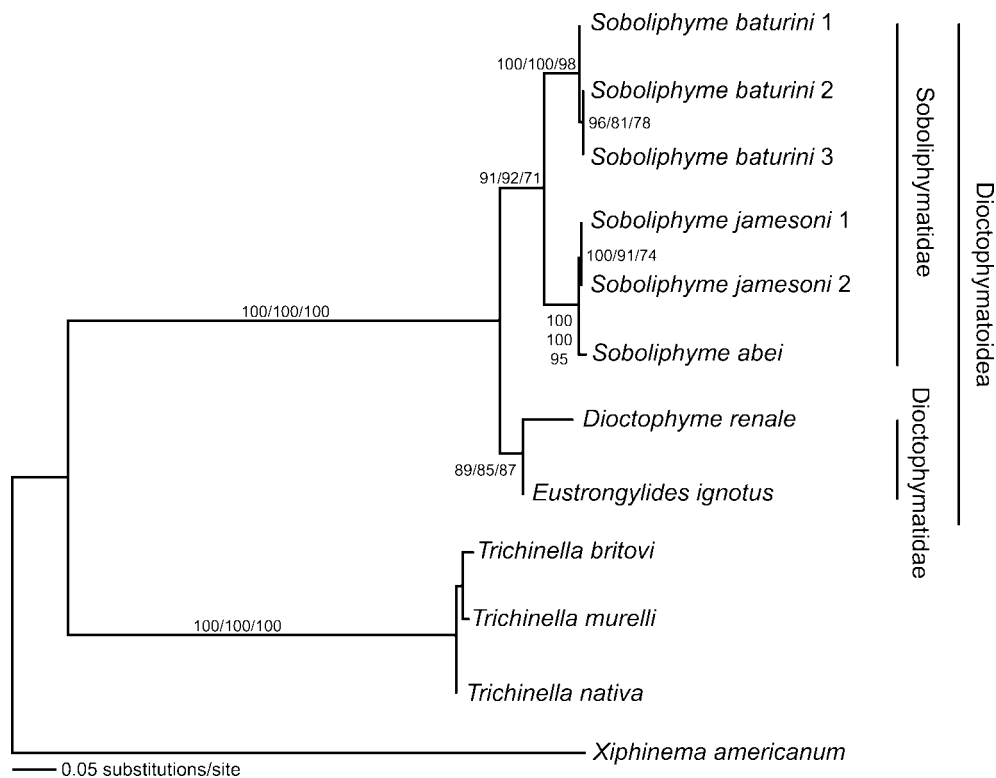
Figure 1. Maximum likelihood tree estimated from partial sequences of the combined 18S and COI genes (1,060 bp) indicates monophyly of 3 genera of the dioctophymatoids and suggests that Soboliphymatidae is sister to Dioctophymatidae. Nodal support values are (left to right): ML bootstrap (1,000 replicates), MP bootstrap (5,000 replicates), and BayesPhylogenies posterior probabilities expressed as percentages. The outgroup is *Xiphinema americanum.*

(AY851256, AB252966), and *Trichinella murrelli* Pozio and LaRosa, 2000 (AY851259, DQ007894) were included to test monophyly of the ingroup.

Both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria were used for phylogenetic reconstruction using PAUP* (Swofford, 2002), considering all characters as unordered with 4 possible states (A, C, G, T). Under both optimality criteria, a branch and bound search was performed using concatenated 18S and COI sequences. A partition homogeneity test (PAUP) resulted in a p-value > 0.05, suggesting that it was appropriate to concatenate the 18S and COI sequences. Node support was evaluated with nonparametric bootstrap methodology using 5,000 replicates for MP (MPB) and 1,000 for ML (MLB) (Felsenstein, 1985). Modeltest v. 3.06 (Posada and Crandall, 1998) was used to determine the appropriate nucleotide substitution model for the concatenated ML matrix, using the Akaike corrected (AICc) option. The model (TIM + I + G) plus invariant sites (I = 0.4041) and

gamma distribution of variable sites (0.3256) was selected as the best model.

The Markov Chain Monte Carlo (MCMC) sampling procedure was performed using the program BayesPhylogenies (Pagel and Meade, 2004) to estimate the posterior probability (PP, expressed as a percentage) of phylogenetic trees. We used a general likelihood–based "mixture model" (MM) based on the general time-reversible model (GTR) of gene-sequence evolution to estimate the likelihood of each tree. To find the best "mixture model" of gene-sequence evolution, we determined the likelihood of the trees by first using a simple GTR matrix, then using a GTR matrix plus the gamma-distributed rate heterogeneity model (GTR + G), and then continuing to add up to 6 GTR + G matrices. We ran 5×10^6 generations and 4 Markov chains, sampling every thousandth tree to assure that successive samples were independent. The first 500 trees were removed to avoid including trees sampled before convergence of the Markov Chain.

Similar topologies were found for each of the phylogenetic analyses (MP, ML, and Bayesian). High support values (MPB = 100, MLB = 100, PP = 100) were found for a clade composed of species of *Dioctophyme*, *Eustrongylides*, and *Soboliphyme*, which is consistent with monophyly of Dioctophymatoidea (Fig. 1). The relationship of the genus *Hystrichis* has yet to be determined with molecular data, but traditional morphological analyses have placed it with the Dioctophymatidae (Karmanova, 1986). Within the Dioctophymatoidea, Soboliphymatidae (species of *Soboliphyme*) is monophyletic (MPB = 92, MLB = 91) and the sister group of the Dioctophymatidae (*Dioctophyme* and *Eustrongylides*; MPB = 85, MLB = 89). These molecular phylogenetic results corroborate prior hypotheses based on comparative morphology (Railliet, 1915; Anderson and Bain, 1982; Karmanova, 1986; Anderson, 2000). Species of *Trichinella* (superfamily Trichinelloidea) included in this study were monophyletic (MPB = 100, MLB = 100, PP = 100) and sister to Dioctophymatoidea, which is congruent with the topology recovered for both clades in the Nematoda phylogeny (Meldal et al., 2007). A comprehensive assessment of diversification that includes broader taxon sampling for all extant species of the Dioctophymatoidea and additional independent genes should be completed. Additional independent genes should also be utilized to resolve relationships at this level.

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